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Molecular Studies of Acute Myeloid Leukemia and the *Telomerase Reverse Transcriptase* Gene

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To the memory of my grandfather Einar Mauritz Lindvall

ABSTRACT

Multiple chromosome rearrangements (MCRs) are detected in approximately 10% of patients with acute myeloid leukemia (AML), and are associated with an adverse prognosis. Comprehensive analysis of the chromosome rearrangements in these complex karyotypes has previously been hampered by the limitations of conventional cytogenetic techniques such as G-banding. As a consequence, our knowledge concerning the genetic alterations in these malignancies is limited. We applied spectral karyotyping (SKY), comparative genomic hybridization (CGH) and cDNA microarrays to bone marrow cells from AML patients with MCRs in order to characterize these rearrangements on the genomic and transcriptional level.

Using SKY and CGH we resolved 12 complex AML karyotypes, and also detected novel chromosome rearrangements. We showed that many of the deletions scored by G-banding harboured cryptic chromosome rearrangements. The majority were unbalanced translocations and most frequently resulted in chromosome loss of 5q, 7q and 17p, and chromosome gain of 11q. In addition, the SKY analysis revealed a number of balanced translocations that had not been described before. Some seem recurrent and may reflect novel fusion genes directly involved in leukemogenesis. (Paper I)

The chimeric transcripts of one reciprocal translocation detected by SKY, a t(8;16)(p11;p13);(*MOZ-CBP*), was analyzed at the sequence level using RT-PCR. The resulting RT-PCR method could be diagnostically useful, since the detection of t(8;16) in AML has clinical ramifications and may be difficult to identify by chromosome banding alone. (Paper II)

In order to investigate the transcriptional profiles of leukemic cells with MCRs we also developed and applied a high-density cDNA microarray assay. We showed that most of the chromosome rearrangements were manifested in aberrant gene expression profiles in a gene-dosage dependent manner. (Paper III)

We also investigated the role of hTERT/telomerase in tumorigenesis and disease. Telomeres, which constitute the ends of chromosomes and are essential for genomic stability and integrity, are synthesized by a ribonucleoprotein reverse transcriptase called telomerase. Telomerase consists of an RNA template, a reverse transcriptase (hTERT) and telomerase associated proteins.

Because hTERT/telomerase activation is critical for cellular immortalization and tumorigenesis we investigated the copy numbers of *hTERT* in human tumors and found that *hTERT* is a frequent target for DNA amplification. (Paper IV)

Chri-du-Chat syndrome (CdCs) is one of the most common human deletion syndromes and results from a deletion of the distal part of chromosome arm 5p, where the *hTERT* gene is located. We showed that a heterozygous loss of *hTERT* is present in CdCs and that hTERT is limiting for telomere maintenance in humans. Therefore, CdCs might be used as a model to study hTERT regulation and telomerase biology in humans. (Paper V)

Certain somatic cells can acquire an immortal phenotype by forced expression of hTERT/telomerase. Treatment with exogenous hTERT has been proposed as a cell-based therapy to allow indefinite expansion of normal human cells without damaging their genomes. However, using cDNA microarrays we showed that the gene expression profile of hTERT-immortalized fibroblasts (hTERT-BJ cells) is significantly different from that of normal mortal fibroblasts. One of the highly expressed genes in the hTERT-BJ cells encodes epiregulin and we found its expression was required to maintain the immortal phenotype. Given the significant difference in gene expression profiles between the normal and hTERT-immortalized fibroblasts and the close relationship between epiregulin and tumorigenesis, we concluded that the use of hTERT for expansion of normal human cells for therapeutic purposes must be approached with great caution. (Paper VI)

PAPERS INCLUDED IN THIS STUDY

This thesis is based on the following papers,
which will be referred to by their Roman numerals.

I. **Charlotta Lindvall**, Magnus Nordenskjöld, Anna Porwit, Magnus Björkholm, Elisabeth Blennow. Molecular Cytogenetic Characterisation of Acute Myeloid Leukaemia and Myelodysplastic Syndromes with Multiple Chromosome Rearrangements. Haematologica. 2001 Nov;86(11):1158-64.

II. Ioannis Panagoloulos, Margareth Isaksson, **Charlotta Lindvall**, Magnus Björkholm, Tomas Ahlgren, Thoas Fioretos, Sverre Heim, Felix Mitelman, Bertil Johansson. RT-PCR Analysis of the *MOZ-CBP* and *CBP-MOZ* Chimeric Transcripts in Acute Myeloid Leukemias With t(8;16)(p11;p13). Genes Chromosomes Cancer. 2000 Aug;28(4):415-24.

III. **Charlotta Lindvall**, Kyle Furge, Magnus Björkholm, Xiang Guo, Elisabeth Blennow, Brian Haab, Magnus Nordenskjöld, Bin Tean Teh. Combined Genetic- and Transcriptional Profiling of Acute Myeloid Leukemia with Complex and Normal Karyotype. Manuscript

IV. Anju Zhang*, Chengyun Zheng*, **Charlotta Lindvall***, Mi Hou, Jessica Ekedahl, Rolf Lewensohn, Zhoungqun Yan, Xiaoyian Yang, Marie Henriksson, Elisabeth Blennow, Magnus Nordenskjöld, Anders Zetterberg, Magnus Björkholm, Astrid Gruber, Dawei Xu. Frequent Amplification of the *Telomerase Reverse Transcriptase* (*hTERT*) Gene in Human Tumors. Cancer Research 2000 Nov 15;60(22):6230-5.

V. Anju Zhang*, Chengyun Zheng*, Mi Hou*, **Charlotta Lindvall**, Fredrik Erlandsson, Magnus Björkholm, Astrid Gruber, Elisabeth Blennow, Dawei Xu. Deletion of the *Telomerase Reverse Transcriptase* Gene and Haploinsufficiency of Telomerase in Cri-du-Chat Syndrome. Submitted for publication.

VI. **Charlotta Lindvall***, Mi Hou*, Toshi Tomurasaki, Chengyun Zheng, Marie Henriksson, Magnus Björkholm, Astrid Gruber, Bin Tean Teh, Magnus Nordenskjöld, Dawei Xu. Molecular Characterization of hTERT-immortalized Human Fibroblasts by Gene Expression Profiling: Activation of the *Epiregulin* Gene. Manuscript.

*Contributed equally to this work

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INTRODUCTION

There are more than 100 distinct types of cancer and even more subtypes. Cancers are classified according to the tissue and cell type from which they arise and can be divided into four major categories: cancers derived from epithelial cells (carcinomas), from connective tissue or muscle cells (sarcomas), from hematopoietic cells and cancers derived from cells of the nervous system. Most cancers originate from a single cell that has undergone a specific genetic change that enables it to outgrow its neighbours (Fearon et al. 1987; Fialkow 1976; Hanahan and Weinberg 2000). However, a single mutation is not sufficient to convert a healthy cell into a cancer cell. Instead, cancer seems to arise by a process in which an initial population of slightly abnormal cells, the descendants of a single mutant ancestor, evolves from a hyperproliferative to a malignant state through successive cycles of mutation and natural selection (Cairns 1975; Nowell 1976; Vogelstein and Kinzler 1993). Because cancer is the outcome of a series of random genetic accidents subject to natural selection, no two cases even of the same variety of the disease are likely to be genetically identical. Nevertheless, several lines of evidence indicate that many of the genetic alterations seen in cancer can be rationalized to reflect a few essential alterations in cell physiology that is common to all cancers, namely: the aquisition of autonomous growth, loss of sensitivity to growth inhibitory signals, escape from programmed cell death, deficiency in DNA repair pathways, unlimited ability of cell division, sustained angiogenesis and the ability for invasive growth and metastasis (Hahn and Weinberg 2002; Hanahan and Weinberg 2000).

There were two aims for the present study. The first was to determine specific genetic alterations in one type of cancer, acute myeloid leukemia. The second was to examine the role of telomerase/hTERT in tumorigenesis.

ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) results from a clonal expansion of myeloid precursor cells in the bone marrow, blood or other tissues (Jaffe et al. 2001). AML includes all forms of acute leukemia derived from the myeloid, monocytic, erythrocytic and megakaryocytic cell lineages (Figure 1), and accounts for 70% of all acute leukemias in the Western world (Jaffe et al. 2001). In Sweden, the incidence is approximately 4/100,000 population per year (Astrom et al. 2000). AML is uncommon in childhood and the median age at diagnosis is 69.5 years (Astrom et al. 2000).

The clinical signs and symptoms of AML are diverse and non-specific, but they are usually directly attributable to the leukemic infiltration of the bone marrow, with resultant cytopenia. Typically, patients present with signs or symptoms of fatigue, hemorrhage, or infections with fever due to decrease in red cells, platelets, or white cells, respectively. AML is an acute disorder because it will cause rapid death of the patient in the absence of appropriate therapeutic intervention.

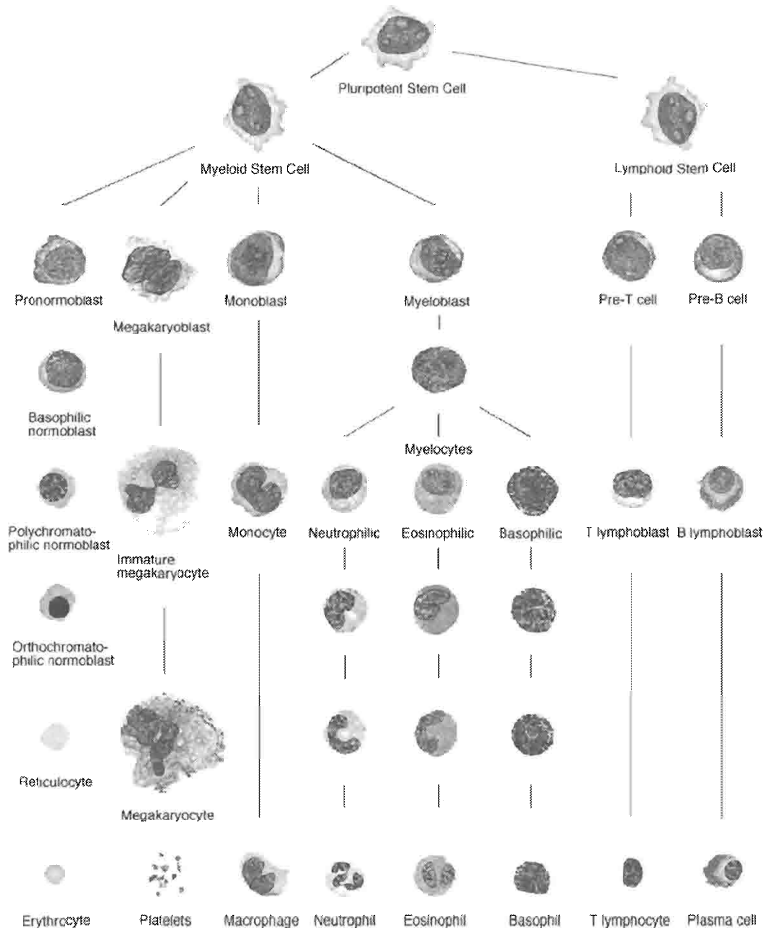

Figure 1. Normal hematopoesis.

Epidemiological studies suggest that both environmental and hereditary factors play a role in the pathogenesis of leukemia. Among the exogenous factors are viruses, ionising radiation, cytotoxic chemotherapy and benzene (Sandler and Ross 1997). Cigarette smoking increases the risk by about two-fold. Certain hereditary disorders including Bloom's syndrome (German 1997; Poppe et al. 2001) and Fanconi's anemia (Butturini et al. 1994) are associated with an increased risk of developing AML. An important feature these disorders have in common is that the susceptibility genes are involved in aspects of recombinational repair of DNA damage (Taylor 2001).

Diagnosis and Classification

Diagnosis of AML is established on the basis of morphological, cytochemical, cytogenetic and immunophenotypic analysis of blood and bone marrow samples. The definitive

criterion for diagnosis is the presence of at least 30% of leukemic blasts in a bone marrow smear (Bennett et al. 1985).

For most of the 20th century, AML was subclassified on the basis of similarities between the leukemic cells and the precursor cells identified at the various developmental stages in normal hematopoiesis. This approach seemed intuitively obvious, and since the treatment of these diseases had poor success, it was quite satisfactory. A cooperative French-American-British (FAB) study group proposed a set of criteria that became widely accepted in AML classification (Bennett et al. 1976, 1985; Cheson et al. 1990). The initial FAB classification from 1976 was based on cytomorphology and cytochemistry. As other techniques like immunophenotyping, cytogenetics, and molecular genetics contributed to the definition of AML subtypes, the classification was updated in 1985 and now encompasses eight major subgroups:

M0 Acute myeloblastic leukemia without maturation
M1 Acute myeloblastic leukemia with minimal maturation
M2 Acute myeloblastic leukemia with maturation
M3 Acute promyelocytic leukemia
M4 Acute myelomonocytic leukemia
M5 Acute monocytic leukemia
M6 Acute erythroleukemia
M7 Acute megakaryoblastic leukemia

A weakness of the FAB classification is its limited clinical relevance. In 1999 the World Health Organization classification for tumors of hematopoietic and lymphoid tissues was proposed (Jaffe et al. 2001). In an attempt to define biologically homogeneous entities with clinical relevance it includes morphologic, immunophenotypic, genetic features and clinical features in its classification. The WHO classification of AML encompasses four major categories:

Acute myloid leukemia with recurrent genetic abnormalities
Acute myloid leukemia with multilineage dysplasia
Acute myloid leukemia, therapy related
Acute myloid leukemia not otherwise categorized

The FAB and WHO classifications are currently used in parallel.

Prognosis

In general, patients with AML have a poor prognosis. About 50-85% of patients will achieve complete remission (<5% of bone marrow blasts) with combined drug therapy. However, most of these patients will relapse, and only 20% will achieve a long-term survival (Hiddemann and Buchner 2001). Adverse prognostic factors include an age over 60 years, poor physical condition before treatment, AML resulting from prior chemotherapy or an antecedent hematologic disorder such as a myelodysplastic syndrome (MDS) and high leukocyte count (Lowenberg 2001). A detailed cytogenetic analysis of the leukemic cells has been demonstrated to provide critical prognostic information (Ferrant et al. 1997;

Grimwade et al. 1998; Heim and Mitelman 1995; Jaffe et al. 2001; Mrozek et al. 2001). AML associated with certain translocations such as t(8;21)(q22;q22), t(15;17)(q22;q12) and inv(16)(p13q22)/t(16;16)(p13;q22) are generally accompanied by a relatively favourable response to therapy, while deletions of 5q or 7q, monosomy 7, inv(3)(q21q26)/t(3;3)(q21;q26) and a complex karyotype are associated with an adverse prognosis. In addition, an assessment for multidrug resistance (van den Heuvel-Eibrink et al. 2000) and immunophenotyping (Hrusak and Porwit-MacDonald 2002) may also provide prognostic information.

Molecular Pathogenesis

Hematopoiesis (Figure 1) is a stepwise process driven by the alternate expression of specific transcriptional regulators, growth factors and growth factor receptors, whose combination determines lineage commitment and maturation (Friedman 2002; Tenen et al. 1997). Regardless of subtype, AML is characterized by a defect in the normal process of maturation that converts a myeloid precursor cell into a mature white blood cell. This block to differentiation is associated with abnormal proliferation, enhanced cell survival and diminished response to apoptotic stimuli (Alcalay et al. 2001; Kitada et al. 2002; Liebermann and Hoffman 2002). Although leukemias are heterogeneous in terms of phenotype, disease progression, prognosis and response to therapy, there are a limited number of mechanisms underlying leukemic transformation. Those that seem to be of major importance in our understanding and management of AML are summarized below.

Cytogenetic abnormalities

Approximately 65% of AML patients have detectable cytogenetic aberrations in their leukemic cell population at the time of diagnosis, the remainder have a normal karyotype (Heim and Mitelman 1995; Grimwade et al. 1998; Mrozek et a. 2001). The cytogenetic aberrations can be divided into three major groups: *balanced rearrangements*, *unbalanced or numerical aberrations*, and *multiple chromosome rearrangements*. However, these groups overlap, for example, a primary translocation can sometimes be seen as part of a complex karyotype.

Balanced rearrangements. The vast majority of cytogenetic aberrations in AML are chromosome translocations and inversions. These balanced rearrangements often represent critical, early events in the genesis of the leukemic clone (Bernardi et al. 2002; Brown et al. 1997; Castilla et al. 1996; Corral et al. 1996; Yuan et al. 2001). At the site of the chromosome break, they give rise to gene fusions. The coding exons of the two genes involved become juxtaposed and form a single fusion gene, which codes for a novel hybrid protein with unique feature. Of the two components of each fusion protein, one is usually a transcription factor, whereas the other partner is more variable in function, but is often involved in the control of cell survival and apoptosis (Alcalay et al. 2001; Look 1997; Scandura et al. 2002). As a consequence, AML-associated fusion proteins function as aberrant transcriptional regulators that interfere with the process of myeloid differentiation and enhance cell survival. In addition, the specific stage of myeloid maturation arrest appears to be directly dependent on the nature of the fusion protein expressed. A large number of diverse translocations have been described in AML, of which several are associated with specific subtypes of AML (Mitelman et al. 2002). The most well studied are t(15;17)(q22;q12);(*PML-RARa*) in M3 acute promyelocytic leukemia (APL) (Brown et al.

1997), t(8;21)(q22;q22);(*AML1-ETO*) in M2 AML (Yuan et al. 2001), and inv(16)(p13q22)/t(16;16)(p13;q22);(*CBFβ-MYH11*) in M4 AML with eosinophilia (Castilla et al. 1996). Together with their variants and the t(9;11)(p22;q23);(*MLL-AF9*) (Corral et al. 1996), they account for approximately 40% of all AML (Look 1997).

Unbalanced or numerical aberrations. Recurrent unbalanced or numerical cytogenetic aberrations are also frequently observed in AML (Heim and Mitelman 1995; Grimwade et al. 1998). The most common are deletions of 5q and 7q, and monosomy 7, but deletions of 9q, 12p and 20q, and trisomy 8 are also seen frequently. The critical genes disrupted by these abnormalities have not been identified.

Multiple chromosome rearrangements. Approximately 10% of AML patients do not have a single chromosome aberration at diagnosis but multiple chromosome rearrangements (MCRs) involving three or more chromosomes (Grimwade et al. 1998; Heim and Mitelman 1995). AML patients with MCRs respond poorly to antileukemic treatment and it is likely that some of these rearrangements contribute to drug resistance and disease progression (Schoch et al. 2001). The MCRs in AML often result in chromosome loss of 5q, 7q and 17p and chromosome gain of 11q and 21q (Lindvall et al. 2001; Van Limbergen et a. 2002; Mrozek et a. 2002; Schoch et al. 2002), but the molecular consequences of MCRs are poorly understood.

Tyrosine kinase oncogenes

Activating mutations in tyrosine kinases resulting in constitutive kinase activity are common in AML (Mecucci et al. 2002). Many of the tyrosine kinase oncogenes are derived from genes, such as *c-Abl*, *c-Fes*, *Flt3*, *c-Fms*, *c-Kit* and *PDGFRß*, that are normally involved in the regulation of hematopoiesis or hematopoietic cell function (Scheijen and Griffin 2002). Despite differences in structure, normal function, or subcellular location, many of the tyrosine kinase oncogenes signal through the same pathways, including the activation of phosphotidylinositol 3-kinases (PI3K), the Ras/Raf/MAP kinases, phospholipase C (PLC) and Signal transducers and activators of transcription (Stats) (Rane and Reddy 2002). It should be pointed out that certain chromosome rearrangements can also cause constitutive kinase activity. For example, the fusion protein c-Abl-Bcr of the t(9;22)(q34;q11) has elevated tyrosine kinase activity (Konopka et al. 1984).

The most striking example of a tyrosine kinase oncogene in AML is *FLT3*. Internal tandem duplications and activating mutations have been found in approximately 20% and 7% of AML patients, respectively (Nakao et al. 1996; Schnittger et al. 2002; Yamamoto et al. 2001). Internal tandem duplications are usually found as an isolated anomaly in patients with normal karyotype and have been associated with an unfavourable prognosis (Meshinchi et al. 2001; Schnittger et al. 2002; Whitman et al. 2001). FLT3 is a receptor tyrosine kinase and appears to mediate cell proliferation and differentiation of hematopoietic stem cells (Molineux et al. 1997; Muench et al. 1995). The importance of FLT3 has led to assesment of selective inhibitors as potential therapy for AML. AG1295 (Levis et al. 2001), AG1296 (Tse et al. 2001), herbimycin A and radicicol (Naoe et al. 2001; Zhao et al. 2000), are molecules under investigation that show great promise and may lead to major advances in the treatment of AML.

Tumor suppressor genes

Homozygous mutations in tumor suppressor genes are rare in AML. Nonetheless, dominant-negative mutations have been reported for a number of tumor suppressor genes, including *C/EBPa* (Lin et al. 1993) and *WT1* (Call et al. 1990; King-Underwood and Pritchard-Jones 1998). C/EBPα is a transcription factor, which is known to be a tumor suppressor and proliferation inhibitor (Lin et al. 1993). Recently it was shown that mutations of the *C/EBPa* gene occur in up to 17% of M2 AML with a normal karyotype (Gombart et al. 2002; Pabst et al. 2001b). Furthermore, certain chimeric proteins formed by chromosome translocations, e.g. *AML1-ETO*, can produce a dominant negative transcription factor that can decrease expression of tumor suppressor genes (Pabst et al. 2001a). Gene expression can also be repressed by epigenetic mechanisms such as promoter hypermethylation, and this may be important in the progression of AML. For example, a high frequency of hypermethylation of the $p15^{INK4B}$ promoter has been reported for AML and an inverse correlation between $p15^{INK4B}$ methylation and overall survival has been observed (Chim et al. 2001; Wong et al. 2000).

Alterations of *p53* (Levine 1997), the "guardian of the genome", are found in AML, most frequently in therapy-related AML and in patients with 17p deletions. In two studies involving 351 AML and MDS patients, 64% of the patients with 17p deletions had a point mutation of the remaining *p53* allele. On the other hand, only 3% without chromosome 17p deletions had a *p53* mutation (Fenaux et al. 1991; Lai et al. 1995).

Secondary leukemia

Secondary AML are those associated with prior radiotherapy, chemotherapy, or that arise from an antecedent hematologic disorder such as MDS. Generally patients with secondary leukemia have a poor prognosis (Dann and Rowe 2001). Chemotherapy-related leukemias were first described in survivors of Hodgkin's disease treated with nitrogen mustard (mechlorethamine), but were later found in patients treated with other alkylating agents (procarbazine, lomustine, chlorambucil), and in patients receiving epipodophyllotoxins (teniposide and etoposide) or other drugs that target topoisomerase II (e.g. anthracyclines) (Dann and Rowe 2001). The vast majority of therapy-related leukemias will occur within 10 years of the chemotherapy and the risk of developing them generally relates to the cumulative dose of the offending agent. The cytogenetic lesions associated with therapy-related leukemias segregate with the class of the antecedent chemotherapy. (Andersen et al. 2002; Dann and Rowe 2001; Olney et al. 2002; Pedersen-Bjergaard et al. 2002; Slovak et al. 2002) Secondary leukemias induced by alkylating agents are most frequently associated with loss of all or part of chromosomes 5 or 7, usually present first with myelodysplasia, and have a long period of latency (5-7 years). AML associated with topoisomerase inhibitors occurs with a shorter latency (~2 years) and most commonly involves the translocation of chromosome band 11q23 (the *MLL* gene locus) to chromosomes 4, 9, and 19, causing the t(4;11)(q21;q23), t(9;11)(p22;q23) and t(11;19)(q23;p13.1) translocations, respectively. Other translocations which commonly occur in therapy-related AML include t(3;21)(q26;q22);(*AML1-EVT1*) and t(8;16)(p11;p13);(*MOZ-CBP*).

Telomeres and telomerase in AML

A high level of telomerase activity (more than 10-fold compared to normal hematopoietic cells) has been obseverd in AML (Counter et al. 1995; Engelhardt et al. 2000; Ohyashiki et al. 1997; Zhang et al. 1996). The level of telomerase activity decreases to normal levels at remission and tends to be higher at relapse (Engelhardt et al. 2000; Ohyashiki et al. 1997; Tatematsu et al. 1996). Patients with high levels of telomerase activity show significantly poorer prognosis compared to those with low telomerase activity (Ohyashiki et al. 1997), indicating that telomerase activity might be a prognostic factor in AML. However, most studies have been performed using mononuclear cells obtained from bone marrow or peripheral blood, and comparison of cells at the similar differentiation stages (e.g. CD34+ cells) will be required in the future.

TELOMERASE BIOLOGY

Maintenance of functional telomeres at chromosome ends is required for the prolonged survival of organisms with linear chromosomes. Telomeres serve to limit the loss of genetic material from chromosome ends that is thought to occur due to incomplete DNA replication by DNA-dependent DNA polymerases. Telomeres protect chromosome ends from degradation, recombination and fusion events (Blackburn 2000; Gasser 2000). In all vertebrates including humans, telomeric sequences are composed of 5'-TTAGGG-3' repeats. The size of telomeric DNA (measured in the germ line), however, varies substantially among species, e.g. human telomeres are 10-15 kb, while laboratory mice (Mus musculus) have telomeres that are much longer (>30 kb) and more heterogeneous (Greider 1996). Telomere lengths also vary, albeit to a lesser extent, among somatic cells within a species. In this case, genotype, cell type and cellular replicative history appear to be important variables (Campisi et al. 2001; Forsyth et al. 2002; Prowse and Greider 1995). In most eukaryotes, telomere sequences are synthesized by a ribonucleoprotein reverse transcriptase called telomerase, which consists of an RNA template (hTER), a reverse transcriptase (hTERT) and telomerase associated proteins (Collins and Mitchell 2002; Forsyth et al. 2002; Greider 1998).

All human somatic cells and most cell lines transcribe and accumulate *hTER*, regardless if they express telomerase activity or not. In contrast, *hTERT* mRNA expression is highly correlated with telomerase activity which suggests that hTERT is the rate-limiting component for telomerase activity and is essential for telomere elongation. (Bodnar et al. 1998; Meyerson et al. 1997; Nakamura et al. 1997)

The Telomere Hypothesis of Cellular Aging and Immortalization

A schematic of the telomere hypothesis of cellular aging and immortalization is shown in Figure 2.

Senescence

In contrast to the mammalian germline and early embryonic cells, most somatic cells do not express telomerase (Forsyth et al. 2002). This poses a problem for dividing cells that is

often referred to as "the end replication problem" (Levy et al. 1992). Because DNA polymerases only synthesize DNA in the 5' to 3' direction and also require a short, labile RNA primer that is removed following replication, 50-200bp of 3' telomeric DNA remains unreplicated at the end of each S phase. Consequently, in the absence of telomerase, telomeres shorten with each cell division. When telomeres reach a critically short length, normal cells irreversibly arrest proliferation and acquire a characteristic enlarged morphology, a high frequency of nuclear abnormalities and stain positively for ß-galactosidase. This response has been termed replicative or cellular senescence (Counter et al. 1992; Mathon and Lloyd 2001; Reddel 1998). Accordingly, ectopic expression of telomerase can prevent telomere erosion and replicative senescence in various human somatic cells, including fibroblasts, retinal epithelial cells and endothelial cells (Bodnar et al. 1998; Vaziri and Benchimol 1998; Yang et al. 1999).

Several lines of evidence suggest that the senescence response evolved to suppress tumorigenesis by preventing the proliferation of cells at risk for neoplastic transformation (Campisi et al. 2001; Harley 2002). Consistent with this idea, normal cells undergo a senescence arrest when faced with a variety of stimuli, all of which have the potential to induce or promote neoplastic transformation. These include certain types and levels of DNA damage and certain mitogen signal transducing oncogenes, such as mutant RAS (Robles and Adami 1998; Wei and Sedivy 1999). Thus, dysfunctional telomeres trigger a fundamental cellular response, which is also triggered by many potentially oncogenic stimuli.

Crisis

The cell-cycle arrest imposed at senescence is maintained by signals that activate the tumor suppressors p53 and retinoblastoma (Rb) (Artandi and DePinho 2000; Hara et al. 1991; Shay et al. 1991). If these pathways are abolished, most human cells proliferate until the telomeres become extremely short, whereupon they enter an unstable state termed crisis (Artandi and DePinho 2000). Cells in crisis attempt to proliferate, but because telomere erosion and chromosome instability are so severe, they frequently undergo apoptosis. A few cells, however, acquire a mutation or epigenetic event that enables them to stabilize their telomeres, most commonly by reactivation of telomerase (Chiu and Harley 1997; Counter et al. 1992; Kim et al. 1994). Such cells can then proliferate indefinitely and resist senescence inducing signals, but are at a greatly increased risk for malignant transformation.

Telomerase activation in tumors

Most human malignant tumors express telomerase; those that do not, stabilize their telomeres by a different mechanism termed alternative lengthening of telomeres (ALT) (Henson et al. 2002). The observation that increased telomerase expression is selected for during the malignant progression of tumors (Kolquist et al. 1998) suggests that telomere stabilization is a requisite step for tumor development and that higher levels of telomerase expression offer a selective advantage to the tumor cell.

In human tumors, telomere stabilization and cell immortalization often involve derepression of *hTERT* (Harley 2002), but the mechanisms through which this is achieved are only partially understood. It was recently shown that the *hTERT* gene is amplified in

certain tumors, indicating that a direct mutational mechanism can be responsible for up-regulating telomerase activity (Zhang et al. 2002; Zhang et al. 2000). Furthermore, the expression of certain oncogenes and tumor virus proteins, can directly up-regulate *hTERT* expression (Klingelhutz et al. 1996; Wang et al. 1998; Wu et al. 1999). This ability seems to be cell-type specific, and correlates with the ability of these oncogenes to immortalize cells. It is therefore possible that telomerase activation during tumor development can also occur indirectly, as a result of oncogene activation or a change in the proliferative or differentiation status of the cell.

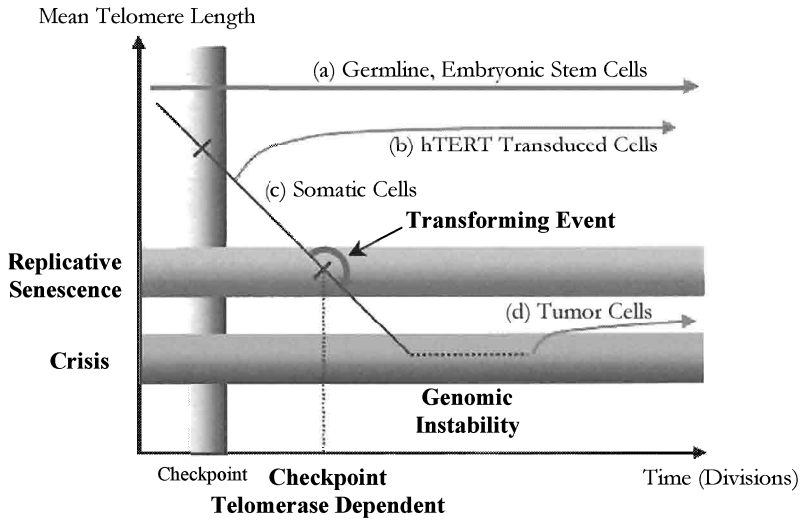

Figure 2. The telomere hypothesis of cellular aging and immortalization. Schematic illustrating telomere maintenance in: (a) immortal germline and embryonic stem cells, achieved by the normal constitutive expression of endogenous telomerase; (b) normal somatic cells immortalized by expression of ectopic (transduced) hTERT; (c) telomerase-negative somatic cells, and (d) tumor cells which have undergone growth control mutations and abnormal activation of endogenous telomerase. In normal, telomerase-negative cells there exist at least two telomere-dependent mortality phases (horizontal bars). Replicative senescence is characterized by a checkpoint-dependent arrest likely triggered by a critical telomere loss or uncapping on one or a few chromosome ends. If cells lack this checkpoint, or suffer a transforming growth control mutation, they can continue to divide, losing telomeric DNA until the crisis phase characterized by major telomere dysfunction, genetic instability, and apoptosis. The vertical bar represents non-telomeric checkpoint arrest mechanisms seen with many human and murine cells placed in culture, or when cells suffer non-lethal acute trauma or inappropriate growth conditions *in vitro* or *in vivo*. (Adapted from Harley 2002)

Balancing Telomere Length

It is paradoxical that both telomere dysfunction and telomerase activation occur in human tumorigenesis. However these events are most likely separated in time during tumor development. Chromosome instability is thought to be initiated early in tumorigenesis (DePinho 2000; Rudolph et al. 2001), whereas telomerase activation probably occurs much later (Chadeneau et al. 1995; Tang et al. 1998). It has been argued that telomerase activation is necessary to inhibit further instability by stabilizing chromosome ends (Hackett and Greider 2002; Maser and DePinho 2002)(Figure 3). However, it is unclear whether telomerase activation in tumors facilitates tumor growth by circumventing checkpoints that recognize dysfunctional telomeres or by stabilizing chromosome rearrangements.

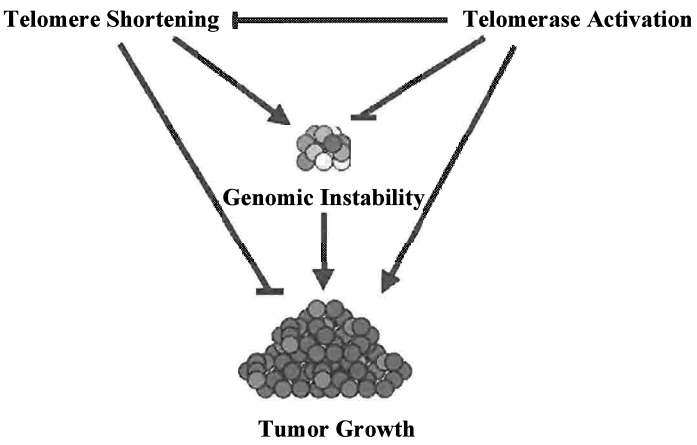

Figure 3. Dual roles for telomerase and telomere dysfunction in genetic instability and tumor growth. Telomere shortening can potentially contribute to the genetic instability that drives tumorigenesis. Early stage tumors with ongoing genetic instability are represented by the cluster of greyish colored balls. However, telomere shortening may also limit tumor growth by activating checkpoints. Telomerase activation can facilitate tumor growth, but telomerase can also help to control genetic instability. Late stage tumors in which certain cells have been selected from early stages in tumor progression are represented by the large cluster of dark-grey balls. (Adapted from Hackett and Greider 2002)

Regulation of the *hTERT* gene

Numerous molecules, including transcription factors, cell cycle regulators, and several viral proteins implicated in tumorigenesis, have been proposed to regulate hTERT expression (Ducrest et al. 2002). Most of these studies were performed by the expression of positive regulators *in vitro* and therefore, the *in vivo* regulation of *hTERT* is still unclear. Nevertheless, studies of the *hTERT* promotor show that it contains a number of putative binding sites for various transcription factors, including c-Myc, Max, Mad and SP1 (Ducrest et al. 2002).

Different *in vitro* studies suggested that c-Myc activates *hTERT* transcription (Greenberg et al. 1999; Wang et al. 1998; Wu et al. 1999). Recently, c-Myc was found associated with the *hTERT* gene *in vivo* in telomerase-positive promyelocytic leukemia HL60 cells using chromatin immunoprecipitation assays (Xu et al. 2001). Differentiation of these cells by DMSO led to down-regulation of *hTERT*, loss of association with c-Myc and binding of the c-Myc antagonist Mad1. Mad is a candidate repressor of *hTERT* and was identified in a gene screen for *hTERT* regulators (Oh et al. 2000). A rise in endogenous *Mad* mRNA and Mad protein levels has been inversely correlated with *hTERT* mRNA levels (Gunes et al. 2000; Oh et al. 2000; Xu et al. 2001). Moreover, wild type p53 has been shown to down-regulate the transcription of *hTERT* in human cancer cells by forming a complex with SP1, thus inhibiting its ability to bind to the *hTERT* promotor (Xu et al. 2000). Since p53 is frequently inactivated in tumors, the loss of *hTERT* repression may be another important consequence of p53 inactivation that promotes tumor development.

Other possible mechanisms of *hTERT* regulation include a putative repressor gene of *hTERT* expression localized to chromosome 3 (Cuthbert et al. 1999; Tanaka et al. 1998), DNA methylation of the *hTERT* promoter (Dessain et al. 2000; Devereux et al. 1999), and alternative splicing (Liu 1999).

Telomerase is critical for human health and viability

Arguably, more is known about telomerase dysregulation than about normal physiological regulatory mechanisms. This may derive in part from a lack of incentive for studying endogenous telomerase regulation in vertebrate model systems such as mice, which survive for many generations in the absence of telomerase (Blasco et al. 1997; Mathon and Lloyd 2001). Still, the impact of telomerase regulation on human health is likely to be much greater than represented by inappropriate activation of telomerase in tumor cells alone.

There is increasing evidence that dysfunctional telomeres may contribute to the development of aging phenotypes, such as atherosclerosis, poor wound healing, and immunosenescence (Effros 1998; Herrera et al. 1999; Klapper et al. 2001; Lee et al. 1998; Rudolph et al. 1999). Furthermore, it was recently shown that *in vivo* mutations in genes encoding two telomerase holoenzyme components reduce the maximal level of telomerase activation and also dramatically compromise the proliferative renewal of hematopoietic and epithelial tissues (Mitchell et al. 1999; Vulliamy et al. 2001). Consequently, human telomerase deficiency may adversely affect normal human development and limit life span (Mitchell et al. 1999; Vulliamy et al. 2001; Zhang et al., unpublished data).

CHARACTERIZATION OF CHROMOSOME ABERRATIONS IN CANCER

The identification of recurrent chromosome aberrations in cancer, and the subsequent characterization of the genes affected by these aberrations, has been one of the great triumphs of molecular biology. A significant part of this success has been due to the rapid development of new molecular technologies. An excellent illustrative example of this is the identification and characterization of the "Philadelphia chromosome" in chronic myeloid leukemia (CML).

In 1960, Nowell and Hungerford reported that cells from CML patients had a normal number of chromosomes, but that one chromosome was too small (Nowell and Hungerford 1960). This small chromosome became known as the Philadelphia (Ph^1) chromosome and was thought to be caused by a simple deletion. Not until the introduction of chromosome banding techniques in the early 1970s, could Rowley show that the Ph^1 chromosome was not caused by a deletion, but an interchange between the end of the long arm of chromosome 9 and the long arm of chromosome 22 (Rowley 1973). It took another 10 years before the translocation was cloned and the disrupted genes identified (de Klein et al. 1982; Groffen et al. 1984; Heisterkamp et al. 1983). On chromsome 9, the translocation disrupts *c-ABL*, the human homologue of the transforming sequence of Abelson murine leukemia virus (A-MuL V), resulting in the expression of an altered form of c-ABL with tyrosine kinase activity (Konopka et al. 1984). Even though the Ph^1 translocation is not the only genetic change associated with CML, the basic research that identified its fusion protein as a tyrosine kinase that is specifically expressed by cancer cells made it an attractive target. Now, two decades later, a drug that specifically target the activated gene product is available for the treatment of CML (Druker et al. 2001). The molecular studies with the Ph^1 chromosome are a scientific success story that began with the identification of a chromosome abnormality and its identification as a translocation. Since the development of chromosome banding techniques, a series of specific chromosome changes that are associated with malignancy, especially in leukemias and lymphomas, have been identified (Heim and Mitelman 1995; Mitelman et al. 2002). Determining the molecular consequence of these changes might result in similar therapeutic strategies as that found for the product of the Ph^1 translocation. This possibility motivates further detailed characterization of chromosome aberrations in cancer.

While it took more than 20 years from the initial discovery of the Ph^1 chromosome until the translocation was cloned, today, chromosome abnormalities can be identified at the molecular level much more quickly. This is mainly due to the development of new cytogenetic techniques such as fluorescent *in situ* hybridization (FISH) technologies (Landegent et al. 1984; Langer et al. 1981; Pinkel et al. 1986) and to the sequencing of the human genome (Lander et al. 2001; Venter et al. 2001)(Figure 4). All FISH technologies are based on the ability of single stranded DNA to anneal or *hybridize* to complementary DNA. In the case of FISH, the target is the nuclear DNA of interphase cells or the DNA of metaphase chromosomes affixed to a glass microscope slide. Recently, matrix based technologies, in which hundreds to thousands of DNA fragments attached to a solid surface serve as the target, were added to the FISH repertoire (Solinas-Toldo et al. 1997). In order to identify and characterize chromosome aberrations using FISH, a variety of probes can be used (Rooney 2001). The three main categories of FISH probes are: probes that identify *a specific chromosome structure*, e.g. probes that bind to telomere and centromere sequences; probes that hybridize to *multiple chromosome sequences*, e.g. spectral karyotyping and comparative genomic hybridization; and probes that hybridize to *unique DNA sequences*, e.g. locus specific probes.

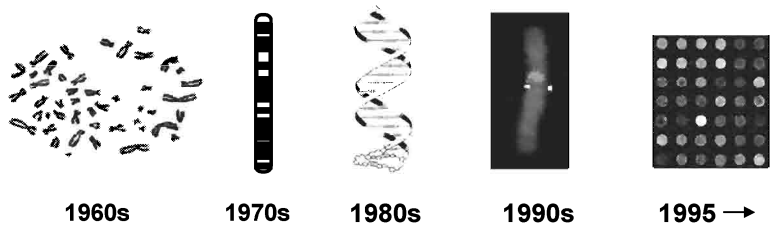

Figure 4. Important steps in the development of genetic technologies. In the 1960s, conventional chromosome analysis became available. With the introduction of chromosome banding techniques in the 1970s it became possible to detect both numerical and structural chromosomal aberrations. During the 1980s, several important molecular techniques were developed, e.g. DNA sequencing and Polymerase Chain Reaction (PCR). In the 1990s, the field of cytogenetics was revolutionized by the introduction of the FISH-methodologies. The latest technical achievement is the development of microarrays, by which thousands of gene or intergenic fragments can be analyzed simultaneously at the DNA or RNA level.

The choice of FISH probes depends, of course, on the biological question in mind. Mapping translocation breakpoints using locus specific FISH probes has proven to be extremely powerful. Locus specific clones, e.g. bacterial artificial chromosomes (BACs), covering a DNA region of interest can be found in public databases such as NCBI (http://www.ncbi.nlm.nih.gov), acquired at a low cost and investigated within a short period of time. In addition, FISH with locus specific probes can be used for many other applications, including identification of gene copy number changes and characterization of chromosome deletions. A prerequisite for these FISH applications is, however, that the researcher has an idea of what to look for in order to choose the right probes. With the development of multiplex (M)-FISH (Speicher et al. 1996) and spectral karyotyping (SKY) (Schrock et al. 1996) (Figure 5), which both involve the simultaneous staining of all the chromosomes in a different color, FISH based screening of the whole genome for chromosome aberrations became possible. M-FISH and SKY are particularly useful in mapping breakpoints, detecting subtle translocations, identifying marker chromosomes and characterizing complex rearrangements (Bayani and Squire 2002; Schrock and Padilla-Nash 2000). Yet, both techniques require metaphase cells, which can be difficult to obtain from primary tumors.

The development of comparative genomic hybridization (CGH) was driven by the need for tools that allow genome wide screening for chromosome aberrations, independently of the availability of metaphase cells in the specimens to be investigated. CGH was introduced by Kallioniemi et al. in 1992 (Kallioniemi et al. 1992), and has been applied to a broad variety of tumor types (Forozan et al. 1997; Lichter et al. 2000; Zitzelsberger et al. 1997). For CGH analysis, whole genomes of test and reference cells are differentially labeled with fluorescent dyes and used as probes for *in situ* hybridization against chromosomes of normal metaphase cells. The differences in fluorescence intensities along the chromosomes in the normal

metaphase are a reflection of the copy number changes of corresponding sequences in the test DNA. The most obvious limitation of CGH is that it only measure differences in copy number, rearrangements not associated with loss or gain of material, such as balanced translocations, are not detectable. Another limitation is the level of resolution. With standard protocols, low copy number gains and losses such as trisomies and deletions are detected only when the imbalanced region is larger than 10 Mb (Bentz et al. 1998; Kallioniemi et al. 1994). Recently, microarray based CGH technologies were introduced which increases the resolution significantly (Pinkel et al. 1998; Pollack et al. 1999; Solinas-Toldo et al. 1997). In matrix CGH, instead of hybridizing to metaphase chromosomes, the reference and test DNA are hybridized against DNA fragments attached to a solid surface, such as a glass microscope slide. The DNA fragments can either be genomic sequences, e.g. BACs, or cDNA clones. The microarrays containing cDNA clones can also be used for gene expression profiling. In fact, they are more commonly used for that purpose.

GENE EXPRESSION PROFILING

Determining the sequence of the human genome has vastly increased our knowledge of genome organization and has been used to identify thousands of previously unknown genes. Various technologies have been developed to exploit this growing body of data, including DNA arrays that provide rapid, parallel surveys of gene expression patterns for hundreds or thousands of genes in a single assay. These transcriptional profiling techniques provide an enormous amount of data that can help us develop a more complete understanding of gene function, regulation and interaction in both health and disease. In cancer research alone, a mounting number of gene expression profiling studies are providing new potential diagnostic and prognostic variables, as well as clues for cancer therapy improvements (Bertucci et al. 2001; Marx 2000).

There are two main types of DNA arrays used for expression profiling: cDNA microarrays containing cDNA clones of approximately 0.5-2 kb length (Schena et al. 1995) and oligonucleotide arrays that use gene specific oligonucleotides in the range of 20-80 bps (Lockhart et al. 1996). The expression profiling experiments in this study were performed with cDNA microarrays (Figure 6).

cDNA microarrays

The first step when planning a cDNA microarray assay is to acquire a well-characterized and annotated set of cDNA clones. After clone selection, amplification and purification, the cDNAs are loaded into microtiter plates of an arraying robot and then mechanically spotted onto chemically modified microscope glass slides. The robotic arrayers provide a reproducible and precise mathematical map from spots on the arrays to wells in the microtiter plates, and therefore, to the cDNA clones and the genes that they represent. Once a collection of microarray slides is printed, each slide can be used for a single experiment.

subsequent agglomeration of clustered pairs into larger clusters, again on the basis of distance (Eisen et al. 1998). Although these methods have proven to be useful for many purposes, e.g. distinguishing novel tumor classes, they also tend to be dominated by shared characteristics of the samples distinguished by large sets of genes. The result is that physiological features of the samples that could in theory be distinguished by variations in the expression of a small set of genes (for example, drug resistance) can in fact be lost in clustering patterns that are dominated by more general aspect of the sample's biology (for example, differentiated versus undifferentiated cell types). To circumvent this, various supervised clustering techniques have been developed as a complement to the unsupervised methods, including Cluster Identification Tool (CIT) (Rhodes et al. 2002). These methods differ in underlying algorithm, but all aim to identify gene expression patterns that are significantly associated with a specific feature of the samples (for example, drug resistance).

Cautionary notes can be made about all microarray analysis methods. The most important being that the identification of a robust structure in a dataset does not necessarily imply biological significance. For instance, samples could cluster according to data quality or method of sample preparation. Such a result might be highly reproducible and statistically significant, but would be of no biological meaning.

AIMS OF THE STUDY

The specific aims of the present study were to:

- Characterize multiple chromosome rearrangements in acute myeloid leukemia on the genomic and transcriptional level.

- Explore copy number changes of the *telomerase reverse transcriptase* gene locus in human tumors and Chri-du-Chat syndrome and correlate *hTERT* copy number to changes in telomerase activity, telomere length and chromosome instability.

- Investigate the molecular consequences of hTERT immortalization of somatic cells.

RESULTS AND DISCUSSION

PAPER I-III: Characterization of chromosome rearrangements in AML

Paper I. In paper I we applied spectral karyotyping (SKY) and comparative genomic hybridization (CGH) to bone marrow cells from 12 acute myeloid leukemia (AML) and 10 myelodysplastic syndromes (MDS) patients with multiple chromosome rearrangements in order to resolve and understand more precisely the genetic alterations in these malignancies.

Multiple chromosome rearrangements (MCRs) can be detected in up to 10% and 30% of *de novo* AML and MDS, respectively, and in up to 50% of therapy-related AML and MDS (Fenaux et al. 1996; Grimwade et al. 1998). AML patients with MCRs have an adverse prognosis. Reported complete remission rates vary from 21-46%, with a median overall survival between 1-5 months (Grimwade et al. 1998; Schoch et al. 2001). Likewise, MDS patients with MCRs tend to progress to AML with a poor prognosis (Greenberg et al. 1997). Although MCRs are frequent in AML and MDS and might be relevant to leukemic transformation and disease progression, it has not been possible to characterize these aberrations in detail by conventional cytogenetic banding techniques such as G-banding. While single chromosome rearrangements can be characterized with considerable precision using G-banding, even skilled cytogeneticists are confronted with great difficulties when attempting to analyze MCRs. The final G-banded karyotypes are often incomplete and exhibit various chromosome abnormalities of unknown origin, as well as deletions and gains of chromosomes. The introduction of multicolor FISH, e.g. SKY (Schrock et al. 1996) (Figure 5), marked a significant methodological improvement making it possible to resolve these karyotypes.

Indeed, our results clearly demonstrated that SKY can resolve complex karyotypes in AML and MDS, and also detect cryptic chromosome rearrangements. In total, 101 structural aberrations were identified by SKY of which only 16 (16%) were possible to characterize to the same extent by G-banding. In general G-banding identified loss of chromosome material quite well, though SKY results often led to a revision of the size of the deleted segment. Furthermore, one-third of deletions scored by G-banding were shown by SKY to represent cryptic translocations or insertions. The vast majority were unbalanced translocations and most frequently resulted in chromosome loss of 5q, 7q and 17p. We also showed that MCRs result in DNA gain of certain chromosome regions. In our patient material, chromosome 8 and chromosome arm 11q showed the most frequent gains. In addition, locus specific FISH for the *MLL* gene revealed that the 11q gains observed resulted in additional copies of *MLL*.

MCRs in AML have often been referred to as random cytogenetic aberrations. However, a number of studies similar to ours have been published suggesting that the MCRs are not random but define a recurring pattern (Kakazu et al. 1999; Kerndrup and Kjeldsen 2001; Mohr et al. 2000; Mrozek et al. 2002; Schoch et al. 2002; Van Limbergen et al. 2002). To summarize, MCRs in AML result in a loss of chromosome material more often than a gain. Unbalanced rearrangements, often represented by unbalanced translocations leading to loss

of chromosome material, are much more frequent than loss of whole chromosomes. The chromosome regions lost are often large and most frequently involve 5q, 7q, 12p and 17p. Structural rearrangements leading to a gain of chromosome material most frequently involve 11q and 21q. Furthermore, gain of 11q results in additional copies of the *MLL* gene. Balanced translocations are also found, but to a much lesser extent than unbalanced translocations. Most of the balanced translocations are novel but some seem to be shared. For example, reciprocal translocations involving chromosome band 4q31 and 5q31 have been reported by three independent groups (Lindvall et al. 2001; Van Limbergen et al. 2002; Veldman et al. 1997). Moreover, certain chromosome aberrations often accompany each other. Deletions of 5q and 17p are frequently seen in the same MCRs karyotype, as are amplifications of 11q and deletions of 17p. Patients with these combined net DNA imbalances appear to have an extremely poor prognosis. Clinical correlations like these represent the most important outcome of these studies, i.e. they indicate that thorough characterization of MCRs in a larger series of patients will make it possible to subdivide AML with MCRs into molecular subclasses, which could reflect different clinical behaviors and prognosis.

Paper II. The aim of paper II was to investigate the resulting chimeric transcripts of the reciprocal translocation, t(8;16)(p11;p13). Case 1 corresponds to patient no. 11 in paper I.

The t(8;16)(p11;p13) results in the fusion of the genes *MOZ* and *CBP* (also named *CREBBP*) at chromosome band 8p11 and 16p13, respectively (Borrow et al. 1996), and is strongly associated with AML displaying monocytic differentiation, erythrophagocytosis by the leukemic cells, poor response to chemotherapy and a dismal prognosis (Becher et al. 1988; Bernstein et al. 1987; Heim et al. 1987; Lai et al. 1987; Quesnel et al. 1993; Velloso et al. 1996). It affects both infants and adults and either arises *de novo*, or is therapy or occupationally related. *MOZ*, which codes for a zinc finger protein with acetyltransferase activity, is also rearranged in other 8p11 aberrations involving *TIF2* in inv(8)(p11q13) (Carapeti et al. 1998) and *p300* in t(8;22)(p11;q13) (Chaffanet et al. 2000). *CBP* codes for a global transcriptional coactivator involved in the regulation of various DNA binding transcription factors (Chan and La Thangue 2001). *MOZ-CPB* fusions may therefore aberrantly activate a number of genes in these AML cases. Heterozygous mutations of the *CBP* gene have been found in patients with Rubinstein-Taybi syndrome, suggesting that haploinsufficiency of CPB may cause this syndrome (Petrij et al. 1995). *CBP* has also been identified as the target of the t(11;16)(q23;p13) in therapy-related AML and MDS, in which it rearranges with *MLL* (Sobulo et al. 1997). In addition, Panagopoulos et al. showed that the *MORF* gene at 10q22, which is highly homologous to *MOZ* (Champagne et al. 1999), was fused to *CBP* in an AML patient with t(10;16)(q22;p13) (Panagopoulos et al. 2001). Together these findings suggest that *CBP* fusions to these histone acetyltransferase genes may play an important role in the pathology of AML.

Previous to our report, only one t(8;16)(p11;p13) was described at the sequence level using reverse transcriptase (RT)-PCR (Borrow et al. 1996). Various explanations for the failure of amplifying the *MOZ-CBP* and *CBP-MOZ* fusion transcripts by RT-PCR were proposed, including low expression or high instability of the transcripts (Giles et al. 1997). However, we showed that both the *MOZ-CBP* and *CBP-MOZ* transcripts can successfully be amplified by RT-PCR in patients with t(8;16). This method could be diagnostically useful,

since the detection of t(8;16) in AML has clinical ramifications and may be difficult to identify by chromosome banding alone. This is particularly true in leukemic cell populations with poor chromosome morphology and/or MCRs. In addition, RT-PCR amplification and subsequent sequencing of t(8;16)(p11;p13) fusion transcripts in more AML patients will enable the detailed genomic characterization of this translocation.

Paper III. In paper III we applied cDNA microarray to compare the global gene expression in mononuclear cells from AML patients with complex (AML-MCRs) and normal (AML-CN) karyotype. All samples were also analyzed by SKY and CGH. Patient AML-MCRs 1, 2, 3 and 4 correspond to patient no. 2, 3, 7 and 13 in paper I, respectively.

A number of research groups have already reported on gene expression profiling of acute leukemias, mostly on acute lymphoid leukemias (ALL) (Armstrong et al. 2002; Ferrando et al. 2002; Golub et al. 1999; Larramendy et al. 2002; Schoch et al. 2002; Virtaneva et al. 2001; Yeoh et al. 2002). The first study from Golub et al. involved the analysis of diagnostic bone marrow samples from AML and ALL patients (Golub et al. 1999). They developed a class prediction algorithm, which could successfully distinguish AML from ALL solely on the basis of the gene expression data. This result was significant not because new methods are required to correctly diagnose ALL and AML but rather because it demonstrated the feasibility of cancer classification based on the analysis of primary tumor samples using DNA arrays and pattern recognition analytical tools. The same group later showed that ALL carrying a chromosome translocation involving the *MLL* gene have a characteristic, highly distinct gene expression profile that can clearly be separated from conventional ALL and AML, suggesting that ALL with *MLL* translocations define a new disease entity (Armstrong et al. 2002). With findings like these in mind, Virtaneva et al. applied clustering and class prediction algorithms to gene expression data from AML samples with isolated trisomy 8 versus normal karyotype (Virtaneva et al. 2001). However, AML with trisomy 8 could not be clearly separated from AML with normal karyotype using the same approaches that successfully distinguished AML, ALL and MLL samples. This was somewhat disappointing but not entirely surprising, since AML with trisomy 8 and AML with normal karyotype are heterogeneous on the phenotypic level and occur in overlapping FAB subgroups (Heim and Mitelman 1995). In contrast, ALL with *MLL* translocations are more homogeneous on the phenotypic level (Jaffe et al. 2001).

In our study we faced the same problem as Virtaneva et al. While the gene expression profiles of both AML-MCRs and AML-CN were clearly distinct from those of samples obtained from healthy bone marrow donors, the two AML groups could not be separated by class prediction and unsupervised clustering algorithms. This result most likely reflects overlapping phenotypes in the patient material, but could potentially be due to limitations of the technology and analytical tools available today. However, by applying a supervised clustering algorithm, Cluster Identification Tool (Rhodes et al. 2002), we could identify individual genes that were differentially expressed between AML-MCRs and AML-CN. Some of these genes were located in chromosome regions we knew contained DNA imbalances in AML-MCRs. This indicated that MCRs might result in an altered gene expression in a gene-dosage dependent manner.

To investigate this hypothesis further, we plotted the expression profiles of all genes mapped to chromosome regions that showed DNA gains and losses by SKY and CGH. Seventeen-75% of genes in amplified regions and 47-88% of genes in deleted regions exhibited a change in gene expression correlated with the type of chromosome aberration. To perform a more objective analysis of whether regions with chromosome gains or losses are associated with a corresponding change in gene expression, we performed a statistical test known as Comparative Genomic Microarray Analysis (material and methods, paper III). This analysis arranges gene expression data based on genomic mapping information and determines if a genomic region (here defined by chromosomal arm boundaries) contains a significantly disproportionate number of genes with increased or reduced expression. We found that most of the chromosome aberrations identified by CGH were manifested in aberrant gene expression profiles as determined by Comparative Genomic Microarray Analysis. To our surprise, regions with a disproportionate number of genes with reduced expression were also found in samples with balanced CGH, most frequently for chromosome arm 7q. These observations need to be further investigated, but the finding is interesting because 7q is frequently deleted in AML and is associated with an adverse prognosis (Grimwade et al. 1998).

Concluding remarks of paper I-III. It has been suggested that the main mechanism of MCRs in leukemogenesis is loss of tumor-suppressor genes since MCRs result in loss of genetic material more often than gain (Schoch et al. 2002). In line with this hypothesis, our microarray data showed that the DNA losses result in an altered gene expression in a gene-dosage dependent manner. However, the SKY and M-FISH reports have also revealed that complex AML karyotypes contain a significant number of balanced translocations, of which the majority are novel and could reflect chimeric genes directly involved in the leukemic transformation. Furthermore, the pathogenic role (if any) of the numerous unbalanced translocations reported by us and others, is at present unknown. The translocated segments might only serve as donors of telomeric sequences necessary to stabilize termini of chromosomes that have undergone terminal deletions, thus do not contribute directly to leukemogenesis. On the other hand, a recent study identified a recurrent unbalanced translocation that resulted in a gene fusion functionally similar to those created by balanced translocations (Ladanyi et al. 2001). Interestingly, at least one of the unbalanced translocations found in our study, der(5)t(5;17)(q11;q11), has also been reported by other groups (Mrozek et al. 2002; Wang et al. 1997).

The complexity of MCRs in AML leads to the assumption of an underlying genetic instability. This instability could in turn explain the poor response to chemotherapy in these patients, because genome instability increases the occurrence of drug resistance. In addition, the mechanisms that allow cells with MCRs to survive instead of undergoing apoptosis may be related to the mechanism that prevents the cells from undergoing apoptosis after cytotoxic treatment. Interestingly, it was recently shown that over-activity of the non-homologous end-joining DNA repair pathways may result in illegitimate joining and alignment of noncontiguous broken DNA ends, leading to translocations, deletions and evasion of apoptosis in myeloid leukemias (Gaymes et al. 2002). However, other mechanisms distinct from impaired DNA repair pathways may also cause MCRs. Studies in yeast have identified numerous genes that, when altered, lead to chromosome instability.

These include genes that function in S-phase checkpoints, recombination pathways, and telomere maintenance (Kolodner et al. 2002).

PAPER IV-V: Consequences of copy number changes of the *hTERT* gene

Paper IV. In paper IV we applied locus specific FISH for the *hTERT* gene to investigate *hTERT* copy number changes in human tumors.

A hallmark of cancer cells is their limitless replicative potential (Hanahan and Weinberg 2000). There is strong selective pressure on tumor cells for replicative immortality, as a large number of cell divisions are required to accumulate the 5-10 independent mutations and clonal expansions typically needed to generate a malignant growth (Harley et al. 1994). In human tumors, telomere stabilization is a requisite step for cells to acquire replicative immortality and often involves derepression of the *hTERT* gene, which is located at 5p15.33 (Bryce et al. 2000; Meyerson et al. 1997; Nakamura et al. 1997). However, the biochemical and genetic mechanisms governing hTERT expression in tumor cells are not well understood. We noticed that in the CGH literature DNA gain of chromosome band 5p15 is frequently reported for various types of tumors (http://www.helsinki.fi/cmg/CHG-data.htm). Because DNA amplification is an important mechanism that allows tumor cells to increase expression of critical genes, we hypothesized that the *hTERT* gene may be a target for DNA amplification.

Our results showed that the *hTERT* gene was amplified in 8 out of 26 (31%) tumor cell lines and 17 out of 58 (30%) primary tumors examined. In addition, 13 out of 26 (50%) cell lines and 13 out of 58 (22%) primary tumors displayed a low copy gain of *hTERT*. For both cell lines and primary tumors the number of *hTERT* copies varied significantly between cells. One neuroblastoma cell line, Lan2, exhibited high-level extra chromosomal amplification of *hTERT* in double minutes. Lan2 showed a 5-fold increase in *hTERT* mRNA expression and a 3-fold increase in telomerase activity compared to cell lines without *hTERT* copy number gain. However, comparing all cell lines, *hTERT* copy number gain could not be significantly correlated to an increased expression of *hTERT* mRNA or increased telomerase activity.

We suggested that acquiring copies of the *hTERT* locus may be one way for evolving tumor cells to increase the level of telomerase, thus ensuring telomere maintenance and replicative immortality. A number of groups have shown that early neoplastic lesions typically possess undetectable or low telomerase activity, whereas the progression to advanced malignant lesions is associated with more robust levels of telomerase (Chadeneau et al. 1995; Engelhardt et al. 1997; Tang et al. 1998; Yan et al. 1999). These findings indicated that increased levels of telomerase is selected for during the malignant progression of tumors. The primary tumors and cell lines in our study exhibited heterogeneity of *hTERT* copy numbers, which may reflect such selective pressure. The variation of *hTERT* copies per cell might explain our difficulties in correlating *hTERT* copy number to *hTERT* mRNA expression, as mRNA was isolated from whole cell populations. However, in a more recent study of cervical carcinomas we could correlate *hTERT* copy number with protein expression by immunohistochemical staining (Zhang et al. 2002).

Paper V. In paper V we investigated whether the 5p deletions present in Chri-du-Chat syndrome are associated with heterozygous loss of *hTERT* and haploinsufficiency of hTERT.

It was recently shown that the hereditary syndrome Dyskeratosis congenita is caused by defective telomerase activity. Mutations in the gene coding for the telomerase RNA template (*hTER*) have been found in the autosomal dominant form (Vulliamy et al. 2001), while the X-linked form is caused by defective processing of *hTER* (Mitchell et al. 1999). These findings led us to the hypothesis that telomerase deficiency might be present in Chri-du-Chat syndrome (CdCs), another hereditary syndrome, which is associated with deletion of chromosome arm 5p (Lejeune et al. 1963).

CdCs is one of the most common human deletion syndromes, with an incidence of 1 in 50,000 births. In young children the syndrome is characterized by psychomotor retardation, microcephaly, growth rate failure, craniofacial abnormalities and micrognathia. One of the most characteristic features in newborn children is a high-pitched cat-like cry that is usually considered diagnostic for the syndrome. (Baccichetti et al. 1988; Gersh et al. 1995; Mainardi et al. 2001; Niebuhr 1978; Overhauser et al. 1994; Van Buggenhout et al. 2000)

We examined the *hTERT* gene status in 10 CdCs patients and found that all exhibited a heterozygous loss of *hTERT*. The deletion of *hTERT* was associated with decreased induction of *hTERT* mRNA expression in activated T-lymphocytes and the lymphocytes exhibited shorter telomeres compared to age-matched controls. A reduction of replicative lifespan and a high rate of chromosome fusions were observed in cultured fibroblasts from CdCs patients. Reconstitution of telomerase activity by ectopic expression of hTERT extended the telomere length, increased population doublings and prevented end-to-end fusions of chromosomes, indicating that restoration of telomerase activity in CdCs fibroblasts can promote genetic stabilization of the cells.

Clinical features of CdCs such as growth retardation, premature gray hair and low sperm production might reflect impaired cell replication caused by haploinsufficiency of hTERT/telomerase. However, it should be kept in mind that the deletions in CdCs vary in size from small, involving only chromosome band 5p15, to the entire short arm of chromosome 5. (Mainardi et al. 2001). Therefore, loss of genes in addition to *hTERT* likely also play crucial roles in the development of CdCs.

PAPER VI: Molecular profiling of hTERT immortalized cells

Paper VI. In paper VI we investigated the molecular consequences of hTERT immortalization of human fibroblasts.

The proliferation of mammalian cells in culture is limited by the development of replicative senescence, first described by Hayflick (Hayflick 1965). Hayflick observed that cultured human fibroblasts exhibit a very reproducible but limited number of cell doublings. Senescent cells arrest their growth, stop cell division, become enlarged and for unknown

reasons, stain positively for β-galactosidase. Replicative senescence is strongly associated with telomere shortening (Mathon and Lloyd 2001) and it was found that introducing hTERT into certain cell types can extend their lifespan and potentially immortalize them (Bodnar et al. 1998; Vaziri and Benchimol 1998; Yang et al. 1999). It has been proposed that treatment with exogenous hTERT might be useful for cell-based therapies by allowing indefinite expansion of normal human cells without damaging their genomes (Jiang et al. 1999; Morales et al. 1999). Even though hTERT immortalized cells lack phenotypic or morphological characteristics of transformed cells, such as loss of contact inhibition or growth in low serum, it is unclear to what whether hTERT-immortalized cells are physiologically and biochemically the same as their normal counterparts. For this reason we compared the gene expression profiles of normal and hTERT-immortalized fibroblasts (hTERT-BJ cells) using high-density cDNA microarrays.

Our results clearly showed that the expression profile of hTERT-BJ cells is significantly different from that of normal mortal fibroblasts. Several genes involved in DNA repair and epidermal differentiation were significantly down-regulated in the hTERT-BJ cells, and considerable expression differences of several growth factors were observed. One of the highly expressed genes in the hTERT-BJ cells encodes epiregulin, a potent growth factor (Baba et al. 2000; Shelly et al. 1998; Shirakata et al. 2000; Toyoda et al. 1997; Zhu et al. 2000). Blockade of epiregulin significantly reduced the growth of hTERT-BJ cells and colony formation of hTERT-transformed fibroblasts on soft agar. In addition, we showed that inhibition of epiregulin function in hTERT-BJ cells triggered a senescence program.

Our results suggested that both activation of telomerase and subsequent induction of epiregulin are required to maintain the hTERT-immortalized phenotype. Given the significant difference in gene expression profiles between the normal and hTERT-immortalized fibroblasts, and the close relationship between epiregulin and tumorigenesis, we concluded that hTERT-immortalized cells should not replace their normal counterparts for studies of normal cell biology. In addition, the use of hTERT for expansion of normal human cells for therapeutic purposes must be approached with great caution.

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